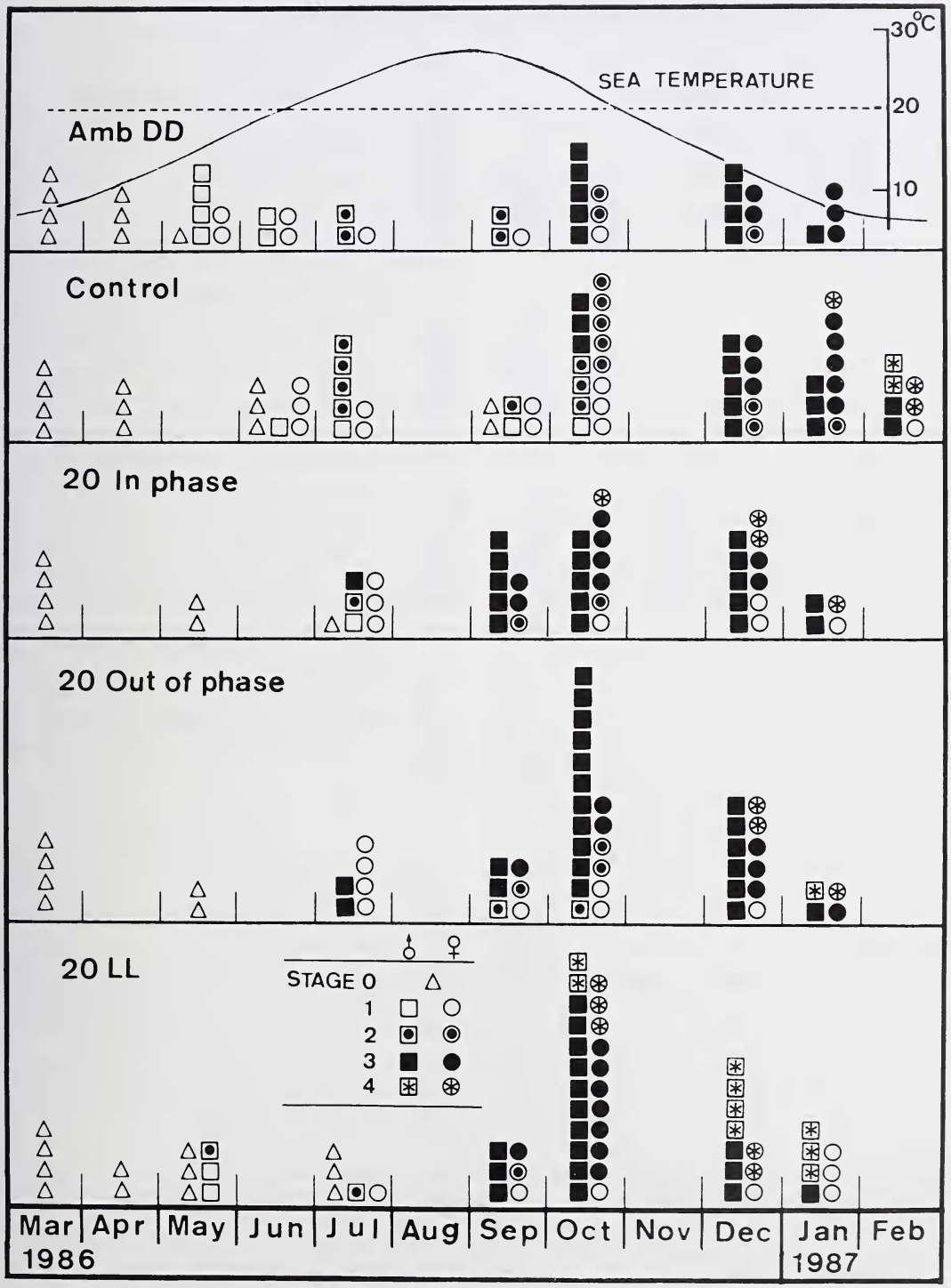

30°C
SEA TEMPERATURE
20
10
Amb DD
Control
20 In phase
20 Out of phase
20 LL
STAGE 0
1
2
3
4
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
1986
1987

Stage 3: *Ovary* — Numerous mature eggs are present in the ovarian cavity (Fig. 1c).
Testis — The lumen is filled with large numbers of spermatozoa (Fig. 2c).

Stage 4: *Ovary* — The ovarian cavity contains only a few relict eggs. Oocytes are few in the ovarian wall (Fig. 1d).
Testis — The lumen is almost empty with a few relict spermatozoa. Spermatogenic cells are few in the testicular wall (Fig. 2d).

Gonadal response

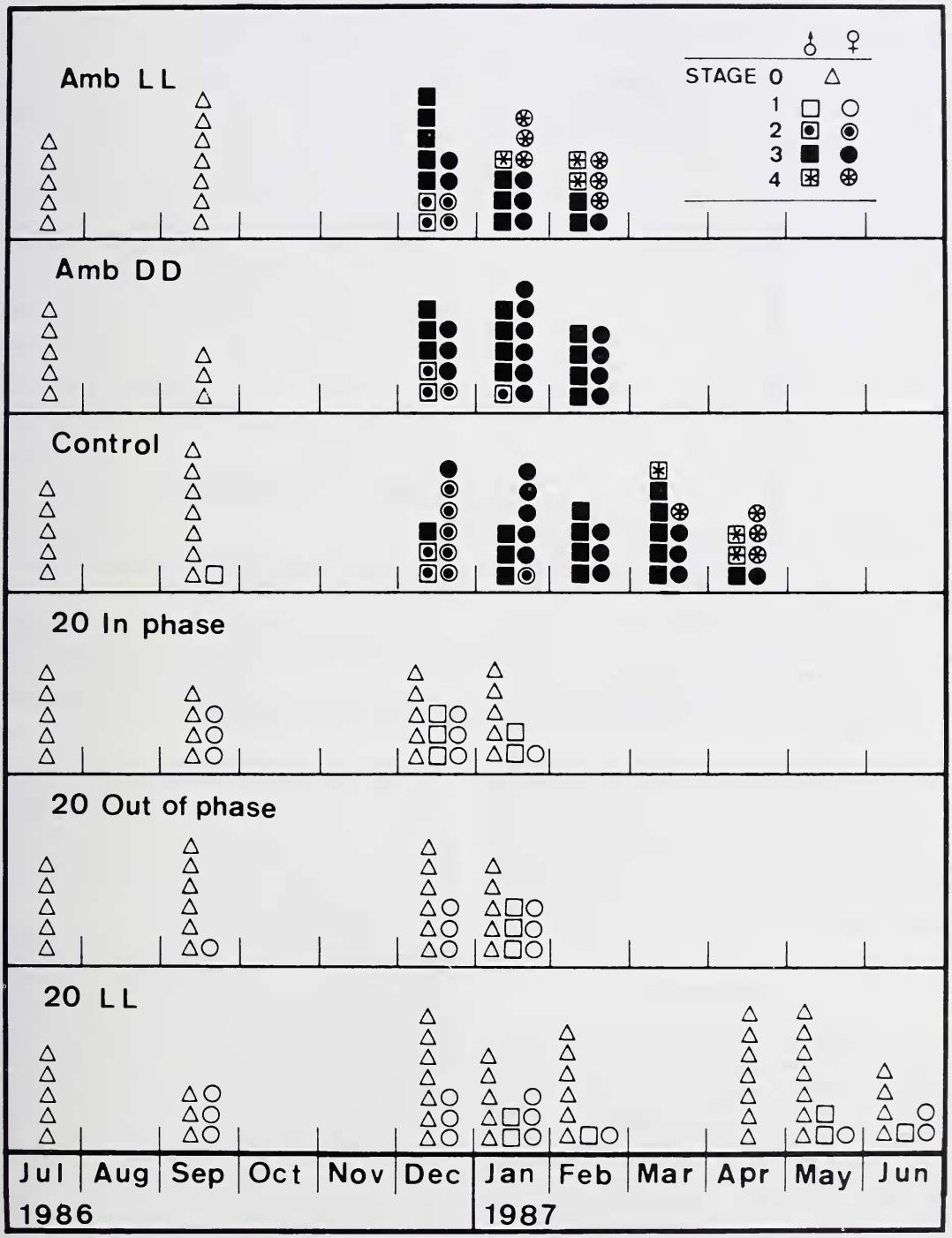
Figure 3 shows the stage of the gonad in each individual of *P. depressus* examined during the course of the experiment. When animals were transferred into the experimental environments in March, all gonads were rudimentary and their sexes cannot be identified from the histological sections (Stage 0). The sexes became evident (Stage 1) in animals larger than about 15 mm in test diameter. In January 1987, the tests had grown to 25–35 mm in diameter and the gonads to 0.6–1.2 g in wet weight of the 5 lobes. We had accidentally lost Groups 2 (20 DD) and 5 (Amb LL) on the way. The gonads in all remaining groups had reached maturity (Stage 3) within a year after fertilization. The testes generally became ripe earlier than the ovaries. In the two ambient temperature groups (Amb DD, Control), the gonads reached Stage 3 almost simultaneously irrespective of the photic conditions. In the three constant temperature groups (20 LL, In-phase, Out-of-phase), the gonads reached Stage 3 earlier than those in the ambient temperature groups (Amb DD, Control), but among these three groups the timing of gonadal maturation is almost identical irrespective of the photic conditions. The gonads in the continuously illuminated group (20 LL) entered the post-spawned stage (Stage 4) earlier than those in the groups under photoperiodic regimes (In-phase, Out-of-phase).

Figure 4 shows the stage of the gonad in each individual of *H. pulcherrimus* examined during the course of the experiment. We had lost Group 2 (20 DD) on the way by an accident. In February 1987, the tests had grown to about 20 mm in diameter and the gonads had grown from a rudimentary state in July to about 0.6–1.0 g in wet weight of the 5 lobes. In the three ambient temperature groups (Amb LL, Amb DD, Control) the ovaries and the testes had reached the maturity (Stage 3) in some individuals in December and in almost all individuals in January irrespective of the photic conditions. Again in *H. pulcherrimus*, the gonads in the continuously illuminated animals (Amb LL) entered post-spawned stage (Stage 4) earlier than those in animals kept under continuous darkness (Amb DD) or ambient daily photoperiod (Control). In the three groups kept at 20°C (20 LL, In-phase, Out-of-phase), the gonads never developed over Stage 1 at least up to January; the gonads were as large as or rather a little larger than those in the ambient temperature groups but they were full of nutritive cells and in many animals even the sexes cannot be identified (Stage 0). Some animals were kept under continuous light (20 LL) until June 1987 but their gonads remained at Stage 0 or 1.

Body growth

Pearse *et al.* [15] have reported in *Strongylocentrotus purpuratus* that the peak in body growth rate could be sifted 6 months in animals kept under 6 months out-of-phase photoperiodic regime. We compared the growth rates among three groups kept under three different light regimes at a constant water temperature of 20°C (20 LL, In-phase, Out-of-phase). In *P. depressus*, the group kept under continuous light (20 LL) grew significantly faster than groups under in-phase and

Fig. 4. The stages of the gonads of *Hemicentrotus pulcherrimus* maintained under 6 experimental conditions. Each symbol represents one individual. Sea urchins were reared from zygotes of the same batch fertilized on 29 March 1986 and were kept under constant illumination at 20°C until they were transferred to one of the following experimental environments on 21 June 1986: continuous light at 20°C (20 LL); changing photoperiod in phase with ambient daily photoperiod at 20°C (In-phase); changing photoperiod 6 months out of phase with ambient daily photoperiod at 20°C (Out-of-phase); continuous light at ambient temperatures (Amb LL); continuous darkness at ambient temperatures (Amb DD); ambient light at ambient temperatures (Control).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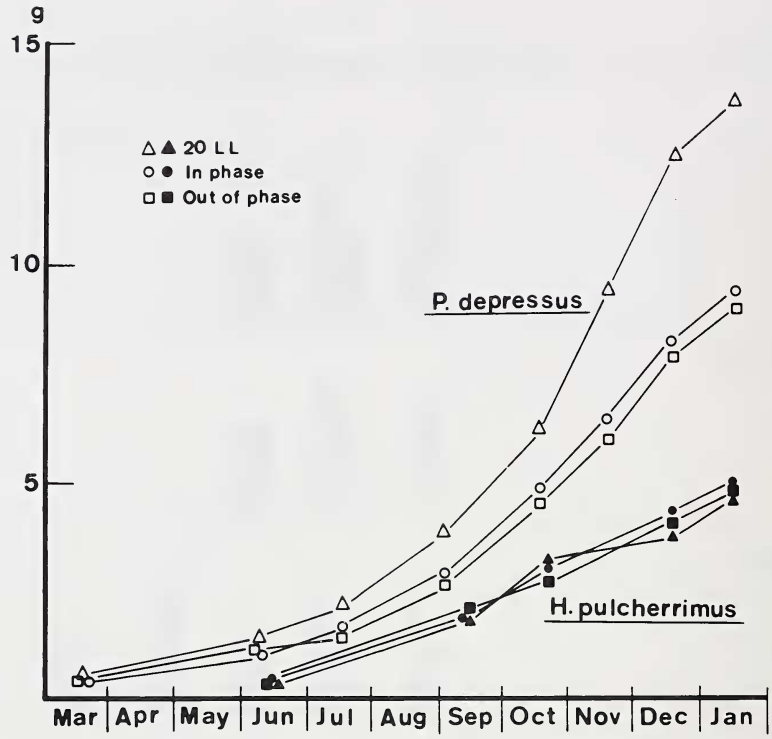

Fig. 5. The wet weight of *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* maintained under three different light regimes at 20°C: continuous light (20 LL), changing photoperiod in phase with ambient ones (In-phase) and changing photoperiod 6 months out-of-phase with ambient ones (Out-of-phase). Each point represents the mean of all individuals maintained, which were about 75 at the beginning of the experiment but decreased in number by being sampled for histological analysis during the experiments.

6 months out-of-phase photoperiodic regimes but the latter two groups grew at the same rate throughout the experimental period (Fig. 5). In *H. pulcherrimus*, the growth rates did not differ among the three groups under the different light regimes (Fig. 5).

DISCUSSION

It is difficult to evaluate the contribution of environmental factors to gonadal maturation unless the conditions of animals prior to the experiment were fully evident. Boolootian [12] has reported in *S. purpuratus* that a population maintained in a constant laboratory condition remained reproductive synchrony with the field ones over three years. Pearse *et al.* [20] suggested in the sea star, *Pisaster ochraceus*, the presence of an endogenous circannual rhythm that is set very early in life and insensitive to experimentally fixed daylengths. In the present experiments we used sea urchins derived from fertilized eggs of the same batch and reared under a constant environment (continuous light at 20°C) until they were transferred to experimental environments.

The experiments by Pearse *et al.* [15] have showed that in *Strongylocentrotus purpuratus,* both gametogenesis and body growth are regulated by seasonal changes in photoperiod. The normal winter to spring spawning period of this species could be shifted to summer to fall by exposing sea urchins to changing photoperiod set 6 months out of phase with ambient daily photoperiod. Gametogenesis in *S. purpuratus* is also responsive to fixed photoperiod [16]. After one year maintenance under fixed short days (8L: 16D) or fixed neutral days (12L: 12D) the gonads became ripe, but under fixed long days (16L: 8D) the gonads did

not have gametes.

The present experiments show that in two common Japanese sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, gametogenesis is under different environmental control from that in *S. purpuratus*; no specific photic conditions are required for gametogenesis to proceed. In *H. pulcherrimus*, the timings of gonadal maturation were identical among the three groups kept under different photic conditions (continuous darkness, continuous light and ambient photoperiod) as long as the animals were kept at ambient temperatures. In *P. depressus* kept at ambient temperatures, the groups under continuous darkness and ambient photoperiod had mature gonads simultaneously. Moreover, in *P. depressus* kept at 20°C, the gonads reached the maturity at the same time in three groups under different light regimes (continuous light, in-phase and out-of-phase photoperiod). These results indicate that gametogenesis proceeds independently of environmental photic conditions. In the two species, unlike in *S. purpuratus* [15], growth was also unaffected by difference in photoperiodic regimes, although the continuous light seems to promote the growth in *P. depressus*.

Our experiments suggest that temperature is a factor controlling gametogenesis in *P. depressus* and *H. pulcherrimus*. In all groups of *H. pulcherrimus* maintained at 20°C, the gonads remained undifferentiated or immature after the gonads in the ambient temperature groups became ripe. In contrast, in all groups of *P. depressus* kept at 20°C, the gonads reached maturity within a year after fertilization. These results suggest the existence of a species-specific temperature range permitting the progression of gametogenesis. The effect of temperatures on sea urchin gametogenesis is now being analyzed in the succeeding experiments.

Although photic conditions do not seem to regulate the onset of gametogenesis in the present species, they seem to relate to the end of gametogenesis. In the gonads of both *H. pulcherrimus* and *P. depressus*, entrance to the post-spawned stage was promoted by a continuous illumination. *S. purpuratus* is also a contrast to the present two species in this respect. In the former, cold temperature treatments did not affect the onset of gametogenesis but high temperature treatments induced gamete resorption [13].

The difference in regulatory mechanisms of gametogenesis between the present species and *S. purpuratus* may be ascribed to the difference in the condition of water temperatures in the sea where they live. Orton [21] have argued that sea temperature is the main environmental factor controlling reproductive activity of many shallow-water marine animals. Seasonal changes in sea temperature are marked along the coast of Japan: monthly means of sea water temperature range 9 to 26°C near the Ushimado Marine Laboratory. In contrast, there are only weak and poorly defined seasonal changes in sea temperature on the Pacific coast of California: Monthly means of sea water temperature range 12 to 17°C [20] at Santa Cruz, California where the experiments in *S. purpuratus* by Pearse *et al.* [15] were carried out. In such a condition, the photoperiod can be a main factor to control the reproduction of marine invertebrates. Pearse's group has reported the presence of photoperiodic control of the annual reproductive cycles in two species of sea stars from California [20, 22, 23].

Since the present materials were reared under the constant illumination at 20°C from the beginning of their lives, the experimental groups maintained under continuous light at 20°C (20 LL) appear to be free from any environmental cues to start the gametogenic cycle. In such a group of *P. depressus*, the gonads ripened synchronously at a different time from those in the groups maintained at ambient temperatures. There may be an endogenous temporal program to start the gametogenic cycle.

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Normal Embryonic Stages of the Pygmy Cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann

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ABSTRACT—A normal table of embryonic stages for the pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann, a suitable material for developmental study of the cephalopod, is presented. A female cuttlefish kept in still sea water in a small vessel repeatedly lays 30–80 fertilized eggs every 2-7 days for more than a month. Thirty stages are morphologically defined during the embryonic period from oviposition to hatching. Hatching occurs 16–18 days after oviposition at 20°C. The external shape and some internal structures of the living embryo at each stage are illustrated, and the chronological ages and main developmental events are assembled in a tabular form.

INTRODUCTION

The cephalopod is unique in its developmental pattern: unlike other molluscs, its eggs are telolecithal, show no spiral cleavage and develop directly to miniature adult forms. However the developmental processes are not fully analyzed due to the difficulty of obtaining eggs, especially at early stages of development. At present, *Loligo pealii* [1, 2] and *Loligo vulgaris* [3] are mainly used in experimental studies of cephalopod development because their eggs are obtainable comparatively easily. The pygmy cuttlefish, *Idiosepius pygmaeus paradoxus*, can be a more useful experimental organism for the developmental study of the cephalopod. They are widely distributed along the coast of Japan [4]. They are small in size (about 15 mm in mantle length) and are easily collectable by a hand net in the eelgrass bed. The maintenance of adults and obtaining of eggs are very easy [5, 6]; no special equipment is necessary to keep them in the laboratory. They lay eggs repeatedly in small vessels; in this laboratory just laid eggs are available every day for at least half an year. The egg is very transparent and its size is suitable for handling (about 1 mm in longer diameter).

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The cephalopod generally has a long embryonic period; a miniature adult form is gradually completed in the egg capsule. Natsukari [6] has described an outline of the development of *Idiosepius pygmaeus paradoxus* and reported the embryonic period to be 15–17 days at about 20°C. The speed of development is so susceptible to environmental conditions, such as temperature and egg density, that it is almost impossible to designate developmental stages correctly only by chronological means. Thus, the morphological staging of *Idiosepius pygmaeus paradoxus* seems to be useful for future developmental studies of this species.

MATERIALS AND METHODS

Adult pygmy cuttlefish, *Idiosepius pygmaeus paradoxus* Ortmann were collected by a hand net or a tow net of a coarse mesh size in eelgrass beds. Near the Ushimado Marine Laboratory, the cuttlefish can be easily obtained from April to August but they disappear from autumn to winter with regression of the eelgrass beds. The male can be distinguished from the female by the white testis visible through the mantle at the caudal end.

Female cuttlefish were kept in still sea water in plastic vessels (3–4 individuals per vessel of 25 cm in diameter) in a constant temperature room (20 ± 1°C). The water was changed every 2 or 3 days.

Aeration was not necessary. The cuttlefish were usually fed with gammaridian amphipods (mainly *Ampithoe valida*) collected by shaking sea weeds (2–3 amphipods per individual per day.) Many other small crustaceans such as marine and terrestrial isopods, fully grown *Artemia* and marine and fresh water shrimps were usable as foods as long as they are alive and the size is appropriate.

Several pieces of microscopic slides, one surface of which was covered with black adhesive tape, were leaned against the inner wall of the vessel with covered face upward. The cuttlefish usually attach to the under surface of the slide with the adhering gland present on the dorsal side of the mantle. Females begin to lay eggs in several days. The eggs are fertilized when they are laid; females have usually received spermatophores from males in the sea. Two maturation divisions begin successively after oviposition. The eggs, each wrapped with a thin vitelline membrane, several layers of gelatinous coats and a transparent egg-capsule (Fig. 2 1b), are laid into an one-layered compact mass on the under surface of the slide. One female kept laying 30–80 eggs every 2–7 days for more than one month in the laboratory; the duration between two ovipositions varied but the rate of egg production was not only constant in a female but almost equal among females (mean egg production rate (eggs/day) $\pm$ s.d. $= 13.7 \pm 0.4$, n=6).

The slide with eggs on one face was transferred to a petri dish and kept at 20 ± 0.5°C. Development tended to be retarded in the eggs laid in the central area of an egg mass. In order to keep synchronous development among embryos, some eggs were removed from the slide so that no eggs were closely surrounded by others. Developing embryos were periodically observed under a stereoscopic microscope (Nikon, SMZ–10) at the magnification of 40–60. The external shapes and some internal structures observable from the outside were sketched with the aid of drawing apparatus. Preliminary staging was made by comparing more than 10 series of sketches of separate batches. The final staging was constructed by correcting the preliminary one through checking it against the development of further 8 batches. The chronological ages are determined by averaging the time data obtained from 10 developmental series of separate batches.

Explanatory illustrations of organ primordia are presented in Figure 1 for the sake of convenience.

RESULTS AND DISCUSSION

The developmental period of *Idiosepius pygmaeus paradoxus* from oviposition to hatching was

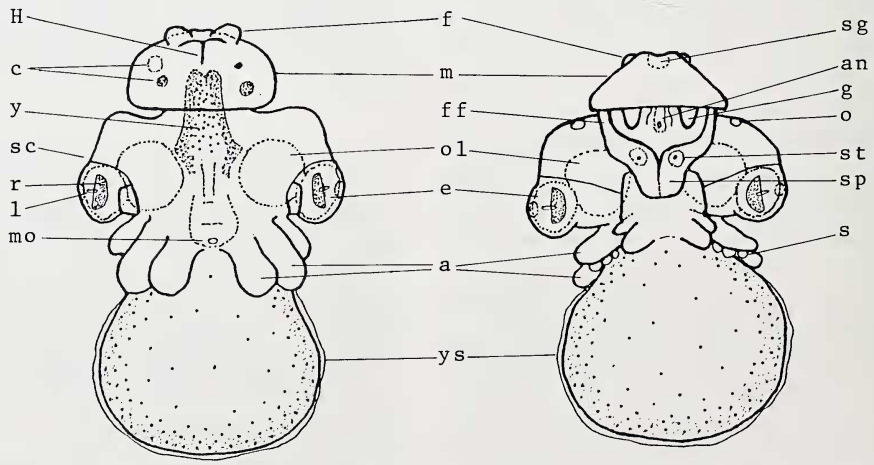

FIG. 1. Organ primordia in the embryo of *Idiosepius pygmaeus paradoxus*. The illustrations are only explanatory and do not represent any particular stages. Left, dorsal view; right, ventral view. a, arm; an; anus; c, chromatophore; e, eye vesicle; f, fin; ff, funnel fold; g, gill; H, organ of Hoyle; l, lens; m, mantle; mo, mouth; o, olfactory organ; ol, optic lobe; r, retina; s, sucker; sc, secondary cornea; sg, shell gland; sp, siphon; st, statocyst; y, yolk; ys, yolk sac.

divided into 30 stages on the basis of morphological features of living embryos. The external shapes and some internal structures of the embryos are illustrated in Figures 2 to 6. Main developmental events to characterize each stage and the mean time to attain the stage after oviposition are presented in Table 1.

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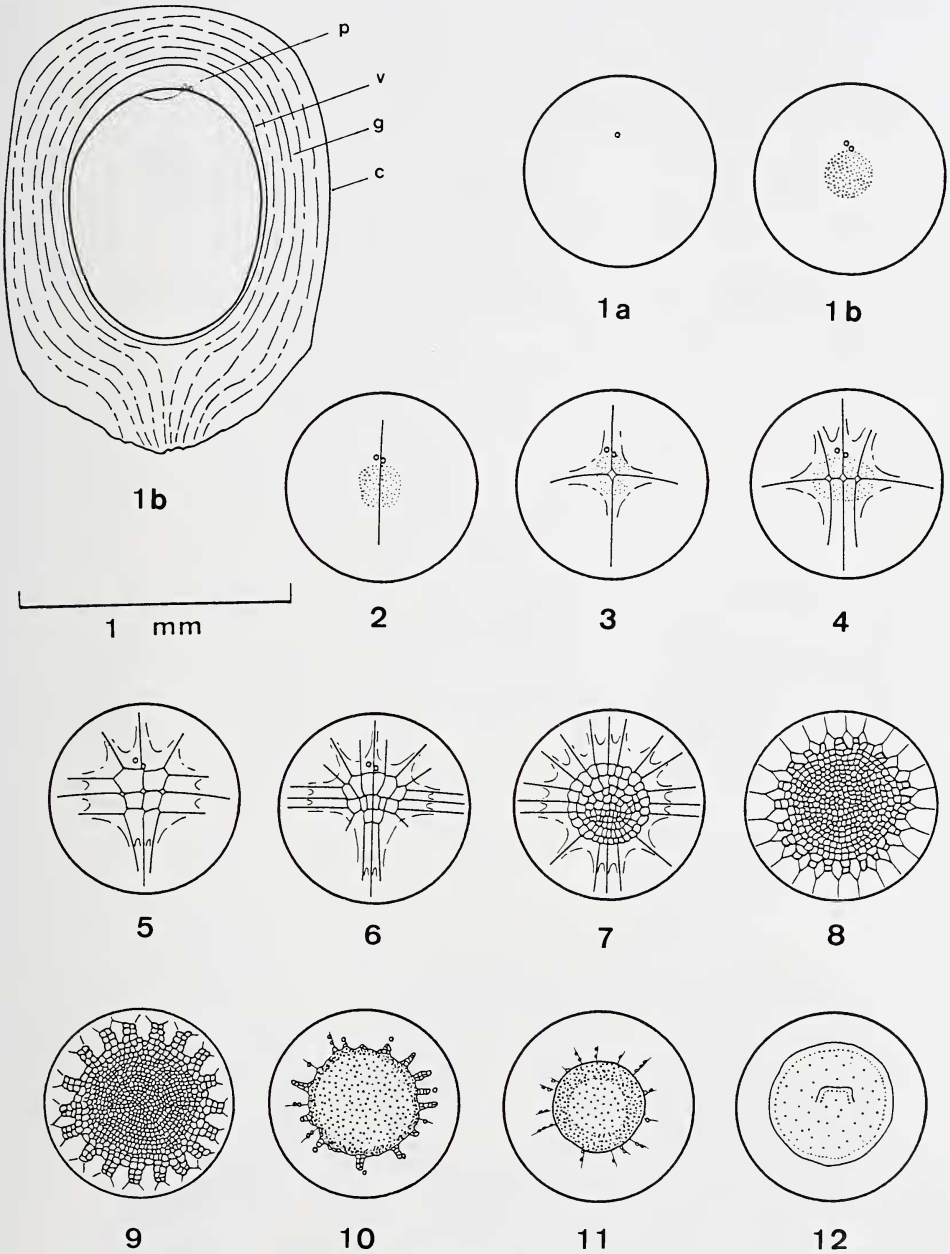

FIG. 2. Normal embryonic stages of *Idiosepius pygmaeus paradoxus*: Stages 1 to 12. The egg envelopes are shown only in one drawing in the upper left, the lateral view of the egg at stage 1b: c, egg capsule; g, gelatinous layer; p, perivitelline space; v, vitelline membrane. The other drawings are views from the animal pole. Each number indicates the stage number.

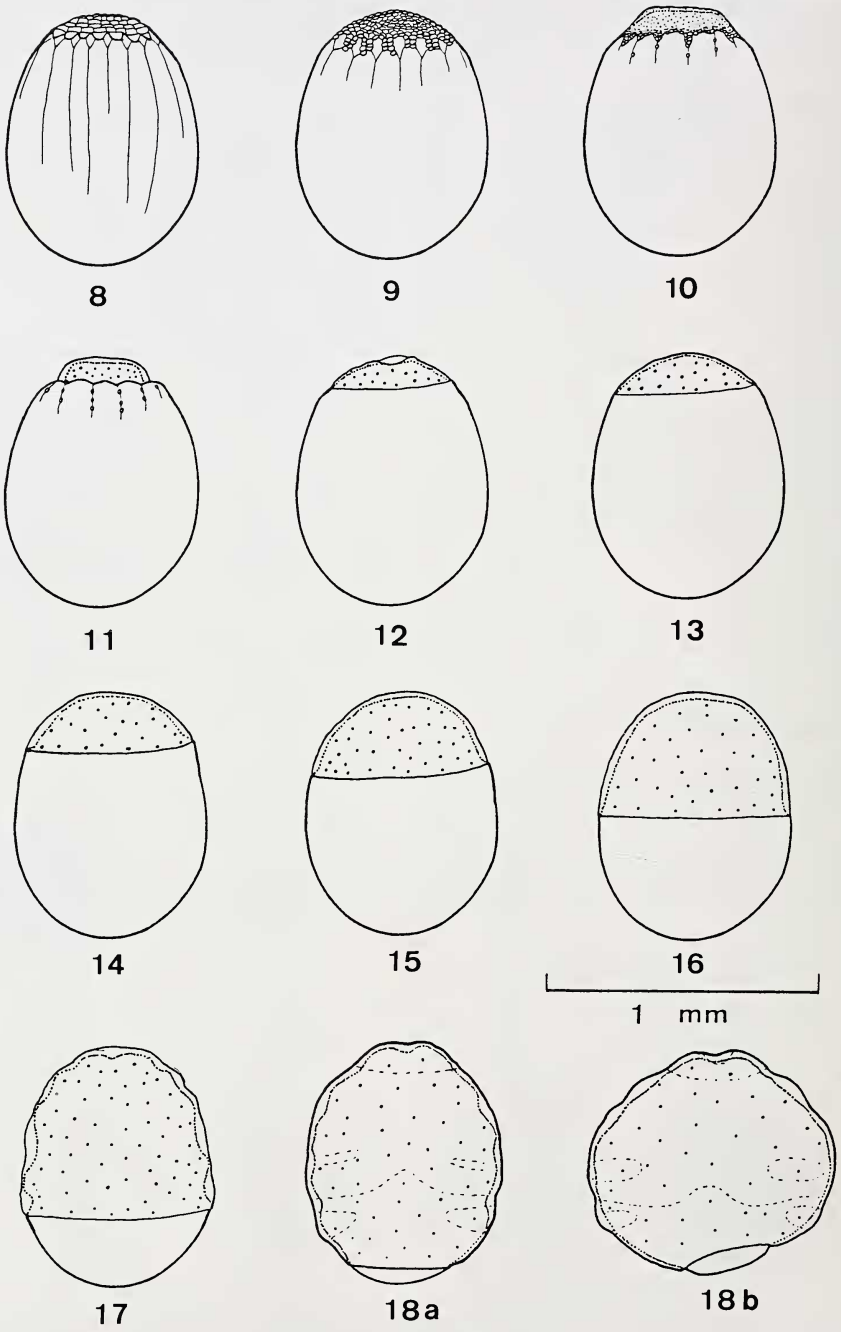

FIG. 3. Normal embryonic stages of *Idiosepius pygmaeus paradoxus*: Stages 8–18. The lateral or dorsal views.